DETECTION AND IDENTIFICATION OF *PRUNE DWARF VIRUS* AND *PLUM POX VIRUS* BY STANDARD AND MULTIPLEX RT-PCR PROBE CAPTURE HYBRIDIZATION (RT-PCR-ELISA)

S.A. Youssef[1,2], A.A. Shalaby[2], H.M. Mazyad[2] and A. Hadidi[1]

[1]*Fruit Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705 USA*
[2]*Plant Pathology Research Institute, Agriculture Research Center, Ministry of Agriculture and Land Reclamation, Giza 12619, Egypt*

SUMMARY

Prune dwarf virus (PDV) and *Plum pox virus* (PPV) are two of the most common viruses infecting stone fruit trees, which are economically important worldwide. To improve the detection of PDV and PPV by PCR technology, we investigated the possibility of detection of these viruses in standard and multiplex RT-PCR-ELISA assays. Total RNA was extracted from cherry, peach, or plum leaves and cherry pollen of PDV-infected or uninfected trees. Total RNA was also extracted from PPV-infected or uninfected leaves. Samples were extracted using commercially available RNA extraction kits. A RT-PCR-ELISA assay was developed for the detection of PDV in a single reaction or with PPV in a multiplex reaction. The use of a PDV-specific capture probe or a PPV-specific capture probe allowed the detection of each virus in a single RT-PCR-ELISA assay. Simultaneous use of the PDV-specific and PPV-specific capture probes permitted the sensitive detection of both viruses in a multiplex RT-PCR-ELISA assay. Nucleotide sequence analyses of the cloned RT-PCR fragments of PDV or PPV obtained from total RNA extracts of infected leaves from different geographical locations revealed > 90% identity with published sequences, which confirmed the identity of each virus isolate investigated.

Key words: PDV, PPV, RNA extraction, detection, RT-PCR-ELISA, amplification, DIG-labeling, cDNA capture probe, nucleotide sequence.

INTRODUCTION

Prune dwarf virus (PDV) and its various strains cause many types of stone fruit diseases of considerable economic importance (Nemeth, 1986). The virus induces considerable damage in many hosts either by itself or in a mixed infection with other stone fruit viruses. PDV is transmitted naturally by infected pollen. PDV has worldwide distribution, especially where sweet and sour cherry are cultivated. Plum pox disease, sharka, is caused by *Plum pox virus* (PPV) (Dunez and Sutic, 1988; Hadidi and Candresse, 2001). Sharka, is the most important viral disease of stone fruit diseases in Europe and the Mediterranean region because of reduced fruit quality, premature fruit drop, rapid natural spread by aphid vectors, and rapid decline and death of trees when co-infected with other viruses. During the last decade, PPV has been reported also from South and North America (Hadidi and Candresse, 2001).

Introduction of PDV and PPV through the international and national movement of stone fruit cultivars and germplasm to local stone fruit industry is of concern to federal and local governments. For this reason, effective control measures of PDV and PPV must be established to safely introduce stone fruit cultivars and/or germplasm that are free of PDV and/or PPV to prevent serious losses to the local stone fruit industry and significantly reduce PDV and/or PPV infection to stone fruits in countries where either virus is present.

Several laboratories, including ours, are involved in developing sensitive reverse transcription-polymerase chain reaction (RT-PCR) assays for PDV and PPV to be used in basic research and by quarantine and certification agencies for viral detection and control (Candresse *et al.*, 1998a; Hadidi and Candresse, 2001). RT-PCR amplified PDV cDNA or PPV cDNA is usually detected by electrophoretic analysis on agarose or polyacrylamide gel followed by gel staining. When a large number of samples has to be processed, gel electrophoretic analysis of the viral amplified product is labor intensive. During the last few years, a combination of RT-PCR and an ELISA type assay in which a probe capture hybridization step is utilized has been described from our laboratory and others for the RT-PCR amplified nucleic acids of viroids (Shamloul and Hadidi, 1999), RNA viruses (Hataya *et al.*, 1994; Weeks *et al.*, 1996; Olmos *et al.*, 1997; Candresse *et al.,* 1998b; Daniels *et al.*, 1998) and for PCR amplified nucleic acids of DNA viruses (Shamloul *et al.,* 2001). With the

Corresponding author: S.A. Youssef
Fax: +202.569.3231
E-mail: saharyoussef@link.net

exception of the report by Candresse *et al.* (1998b) where multiplex RT-PCR-ELISA were utilized for simultaneous detection of *Apple mosaic virus* and *Prunus necrotic ringspot virus*, all the above studies were done using only one virus in the assay. In this paper we report the development of a multiplex RT-PCR-ELISA assay using viral specific primers targeting the coat protein gene of PDV and the 3' non-coding region of PPV coat protein. Both viruses can be simultaneously amplified and digoxigenin (DIG)-labeled in a single PCR reaction. Virus identification is then achieved by microwell capture hybridization assay using PDV or PPV-specific biotin labeled cDNA capture probe.

MATERIALS AND METHODS

Source of PDV and PPV infected tissues. PDV: Egyptian isolates: PDV-B in peach and PDV-M 29 in plum. US isolates: PDV-cherry 37200 kindly provided by H.E. Waterworth; PDV-Rainer cherry, PDV-SIT 35 Bing cherry and PDV SIT 27 Bing cherry were kindly supplied by W.E. Howell and K.C. Eastwell. Virus infected and uninfected leaves and pollen were used.

PPV: The four prototype PPV strains, whose nucleotide sequences had been published, were used: the Egyptian El-Amar; the French D, the Greek M; and the Moldovian sour cherry (C) (Kegler and Hartmann, 1998; Nemchinov *et al.*, 1998). PPV-infected tissues of the four strains were kindly obtained from T. Candresse. Virus infected and uninfected leaves were used.

Total RNA extraction. In most cases, total RNA was extracted from virus- infected or uninfected leaf or pollen tissue using BIO 101 FastRNA Green Protocol (BIO 101, Carlsbad, CA). Occasionally, total RNA was extracted from plant tissue using QIAGEN RNeasy plant mini kit (Qiagen Inc., Valencia, CA) as suggested by the manufacturer.

GeneReleaser treatment of total RNA. One µl of total RNA of each sample was placed in a thin-walled PCR tube containing 23 µl of freshly resuspended GeneReleaser (GR) (Bio Ventures, Inc.,Murfreesboro, TN). The GR-RNA mixtures were vortexed at low speed for 30 sec and held in ice until all samples were prepared. Samples were then placed in a microwave-safe rack (polypropylene, Bio Ventures Inc.), overlaid with 50 µl of mineral oil, lids closed, and microwaved at a high power setting for 6 min.

Primer sequences and the expected size of amplified PDV cDNA or PPV cDNA. Primers for PDV were designed from the nucleotide sequence of the coat protein gene (Bachman *et al.*, 1994) as previously described (Parakh *et al.*, 1995). A23 mer primer (5'-TAG TGC AGG TTA ACC AAA AGG AT-3') complementary to nucleotides 1988-2010 and a 23 mer primer (5'-ATG GAT GGG ATG GAT AAA ATA AT-3') homologous to nucleotides 1838-1860 were designed to amplify a 172 bp cDNA fragment from PDV infected tissue. Primers for PPV were designed to amplify the whole 3' non-translated region of the viral genome (220 bp) as this region is conserved in all known strains of PPV (Hadidi and Levy, 1994; Levy and Hadidi, 1994; Nemchinov and Hadidi, 1996). A 24 mer complementary primer (5'-GTC TCT TGC ACA AGA ACT ATA ACC-3') and a 24 mer homologous primer (5'-GTA GTG GTC TCG GTA TCT ATC ATA-3') were used for amplification. Primers were synthesized by Life Technologies, Inc., Gaithersburg, MD.

Reverse transcription (RT). A 20 µl aliquot of GR matrix containing RNA was removed immediately after microwaveing and added to a primer annealing reaction mixture containing: 6 µl of 5x first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 3 µl of 0.1 M dithiothreitol (DTT), and 1 µg complementary primer. The mixture was vortexed briefly and denatured by heating at 100°C for 5 min, chilled on ice for 2 min and primer annealed at 37°C for 5-30 min or at room temperature for 45 min to allow primer annealing to the viral RNA template. The annealed reaction was added to 20 µl of a cDNA reaction mixture containing: 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl Rnasin (40 units, Promega Corp., Madison, WI), 2 µl of 10 mM dNTPs (2.5 mM each of dGTP, dATP, dTTP and dCTP), and 1 µl of Maloney murine leukemia virus reverse transcriptase (200 U μl^{-1}; Promega Corp.). Reactions were mixed briefly, and incubated for 1-1.5 h at 42°C.

Polymerase chain reaction (PCR). Amplifications were performed in thin-walled PCR tubes and contained the following reaction mixture: 5 µl of 10x PCR buffer (1x = 10 mM Tris- HCl, pH 8.3, 50 mM KCl, and 0.001% gelatin), 3 µl of 25 mM $MgCl_2$ (1.5 mM final concentration), 1 µl of 10 mM dNTPs, 1 µl each of 6 µM complementary and homologous DNA primers, 2.5 units of AmpliTaq Gold^{TM} DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), and sterile H_2O to a volume of 45 µl and 5 µl of cDNA mixture. Each reaction mixture was overlaid with two drops of mineral oil to prevent evaporation during amplification.

Cycling parameters were 13 min at 95°C at the first cycle to activate AmpliTaq Gold^{TM} DNA polymerase,

30 sec at 94°C, 30 sec at 62°C and 45 sec at 72°C for 30 cycles with final extension at 72°C for 7 min in a DNA thermal cycler (Pekin- Elmer Cetus Corp).

Cycling parameters for multiplex PCR for amplification of both PDV cDNA and PPV cDNA were similar to standard PCR except that the DNA polymerase was activated at 94°C for 12 min. In some experiments with multiplex PCR, a gradient of different annealing temperatures (60, 59, 57, 55, or 53°C) were used. These experiments were conducted in a Hybaid thermal cycler (Hybaid Inc., Franklin, MA).

PCR amplification-DIG labeling of PDV cDNA and/or PPV cDNA. PCR-DIG labeling mixtures each contained 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 5 µl of 2 mM dATP, dCTP, dGTP, 5.7 mM dUTP and 0.3 mM DIG-11-dUTP, 2 µl of uracil DNA glycosylase (1 U $µl^{-1}$), 1 µl of 6 mM complementary and homologous primers, 2.5 units of AmpliTaq Gold™ DNA polymerase, and sterile water to a volume of 48 µl. Two microliters of cDNA mixture were added to the PCR reaction and the mixture was covered with 50 µl of mineral oil. The mixtures were amplified with the following cycling parameters: 95°C for 14 min at first cycle, 94°C for 1 min, 60°C for 1 min, 72°C for 2 min for 35 cycles with a final extension at 72°C for 7 min. The PCR cycling parameters for multiplex DIG-labeling of PDV cDNA and PPV cDNA were: 94°C for 12 min at first cycle, 94°C for 45 sec, 60°C or (60, 59, 57, 55, or 53°C) for 1 min, 72°C for 2 min for 35 cycles with final extension at 72°C for 7 min.

Electrophoretic analysis of amplified products. Aliquots (5 µl each) of amplified products were analyzed by electrophoresis on 5% polyacrylamide gels at 100-120 V for 1.5 h in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3) and visualized by staining with silver nitrate. BioLow DNA molecular weight marker (Bio Ventures, Inc.) was used to determine the size of amplified products.

Biotin-labeled PDV cDNA and PPV cDNA capture probes. Biotin-labeled PDV cDNA, 27 oligonucleotides in length, (5'-BIO-TGATTGTGCTTCCACTATGAG-TATTCC-3') was used as a capture probe for products amplified from PDV-infected tissue. PPV cDNA, 23 oligonucleotides in length, (5'-BIO-AGG CCC TTG TAT CTG ATG TAG CG-3') was used as the capture probe for products amplified from PPV-infected tissue. Probes were synthesized and biotinylated at Life Technologies, Inc. The sequence of each probe was selected by using the primer analysis software (rawprimer) from University of Wisconsin, Madison.

Microwell capture hybridization assay. The detection of DIG-labeled amplified DNA was carried out using the PCR-ELISA Detection System (Boehringer Mannheim Corp., Indianapolis, IN) essentially as described by Shamloul and Hadidi, 1999. Briefly, five microliters of RT-PCR-DIG-labeled amplified product were mixed with 20 µl of 0.25 M NaOH then chilled on ice for 2 min. The mixtures were kept at room temperature for 10 min, and then 200 µl of hybridization solution containing 50 ng ml^{-1} 5'- biotinylated DNA capture probe were added. Two hundred microliters of each mixture were pipetted into an ELISA microtiter plate well coated with streptavidin, then the plate was covered with self adhesive tape (3MScotch™, St. Paul, MN) and kept in a shaker at 50°C for 3 h. The hybridization solution was removed and the wells were washed five times with washing PBS-Tween solution (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.1 mM Na_2 EDTA, pH 6.8, 0.05% Tween-20). Two hundred microliters of polyclonal anti-DIG Fab fragment conjugated to peroxidase diluted 1:100 in Tris- HCl, pH 7.5 buffer were added to each well and the microtiter plates were shaken gently at 37°C for 30 min. The Tris-HCl buffer was included in the Detection System obtained from Boehringer Mannheim Corp. Wells were then washed five times with the washing solution. Two hundred microliters of substrate solution (100 µg ml^{-1} of 2,2'- azino-bis {3-ethylbenzthiazoline-6-sulfonic acid} diammonium) were added to each well and microtiterplates were incubated for 0.5-1.5 h at 37°C in the dark with agitation. Solution containing hybridized products was green in color. The absorbency of hybridized products were measured at 405 nm in an ELISA-reader (Multiskan Plus-MK II314). Results were expressed as net absorbance after the optical density of the blank solution was automatically subtracted for each well.

Cloning and nucleotide sequencing of RT-PCR amplified PDV cDNA and PPV cDNA products. The 172 bp PDVcDNA and 220 bp PPV cDNA amplified products were directly cloned into the pCR™ vector using the TA™ Cloning system (Invitrogen, Carlsbad, CA). The ligation mixtures were then used for electroporation of *E. coli* BL21 cells. Recombinant plasmids were finally selected and sequenced. Both strands of each DNA fragment were sequenced by ABI-PRISM™ 373A Genetic Analyzer (Perkin-Elmer) by using dye-primer and dye-terminator methods at University of Maryland College Park, MD (DNA Sequencing Facility/Center for Agricultural Biotechnology).

RESULTS

Analysis of DIG-labeled PDV amplified products. Unlabeled and DIG-labeled RT-PCR amplified PDV cDNAs from leaves or pollen of PDV - infected cherry, peach, and plum isolates of PDV were analyzed by polyacrylamide gel electrophoresis (Fig. 1). The size of the unlabeled amplified PDV cDNA was as expected 172 bp (lanes 1, 3, 5, 7, 9, 11, and 13). The electrophoretic mobility of the DIG- labeled PDV cDNA was relatively slower than that of its respective unlabeled cDNA (lanes 2, 4, 6, 8, 10, 12, and 14). No products were amplified from samples of healthy plants or buffer controls (not shown).

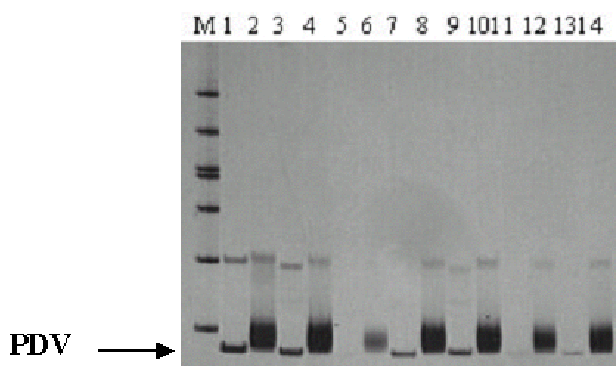

Fig. 1. Gel electrophoretic analysis of RT-PCR (lanes 1, 3, 5, 7, 9, 11, and 13) and DIG-labeled RT-PCR (lanes 2, 4, 6, 8, 10, 12, and 14) product amplified from GeneReleaser -treated total RNA from PDV-infected tissues. Molecular DNA marker with fragment sizes (bp) of 1000, 700, 525, 500, 400, 300, 200, (100, 50, not shown) (M). RT-PCR and DIG-labeled RT-PCR products amplified from leaves or pollen of: PDV cherry 37200 A (lanes 1and 2), PDV peach B (lanes 3 and 4), PDV plum M29 (lanes 5 and 6), PDV Rainer cherry (pollen) (lanes 7 and 8), PDV Bing cherry SIT 35 (pollen and leaves) (lanes 9,10 and 11, 12, respectively), PDV Bing cherry SIT 27 (lanes 13 and 14). Arrow indicates the position of unlabeled and DIG-labeled amplified PDVcDNA.

Detection of PDV DIG-labeled RT-PCR products using PDV-specific capture probe in a microwell capture hybridization assay. DIG-labeled PDV cDNAs were analyzed by capture hybridization assay using biotinylated PDV cDNA probe (Table 1). All infected tissue samples showed positive RT-PCR-ELISA assay with absorbance values at 405 nm ranges from 2.908 to 0.459. All infected samples were green in color. Color development was absent with products from healthy tissue or buffer control samples and their absorbance values were less than 0.025. The sensitivity of detection of PDV DIG-labeled RT-PCR product by probe capture hybridization as compared to gel electrophoretic analysis was 10-100 fold (not shown).

Detection and analysis of DIG-labeled PPV cDNA by probe capture hybridization assay using biotinylated PPV cDNA probe. All known four subgroups of PPV were detected by this method (not shown). Table 2 shows that RT-PCR- ELISA of PPV-El-Amar subgroup was at least 100 fold more sensitive than analysis of amplified products by polyacrylamide gels. Similar results were also obtained with PPV-D, PPV-M, and PPV-C subgroups.

Analysis of unlabeled and DIG-labeled PDV cDNA and PPV cDNA in a multiplex RT-PCR and standard or multiplex RT-PCR-ELISA assays, respectively. Fig. 2 shows the size of the unlabeled amplified PDV cDNA (172 bp) and PPV cDNA (220 bp). The electrophoretic mobility of the DIG-labeled cDNA of each virus was relatively slower than that of its respective unlabeled cDNA (not shown).

In experiments designed to test probe specificity during hybridization in standard RT-PCR-ELISA assays for PDV or PPV, each of the biotin labeled PDV cDNA and PPV cDNA capture probes hybridized only to its respective complementary DIG-labeled RT-PCR amplified product (Table 3). Thus each virus cDNA capture probe is specific.

Table 1. Analysis of PDV isolates by RT-PCR-ELISA.

PDV isolate, source	Name, host, and tissue	RT-PCR-ELISA absorbance at 405 nm
Giza, Egypt	B, peach, leaves	1.095
Giza, Egypt	M29, plum, leaves	0.459
Beltsville, USA	37200, cherry, leaves	2.908
Prosser, USA	rainer, cherry, pollen	2.641
Prosser, USA	SII 35 Bing, cherry, pollen	2.085
Prosser, USA	SII 35 Bing, cherry, leaves	1.538
Prosser, USA	SII 27 Bing, cherry, leaves	1.525
Egypt	uninfected peach or plum leaves	≤ 0.025
USA	uninfected cherry leaves or pollen	≤ 0.025
Buffer	colorimetric substrate	≤ 0.025

Table 2. Sensitivity of detection of PPV El-Amar amplified product by polyacrylamide gel as compared to PCR-ELISA.

Dilution of PPV-infected host RNA extracted by Qiagen method	Analysis of amplified product	
	Polyacrylamide gel	RT-PCR- ELISA
Undiluted	+	+
10^{-1}	+	+
10^{-2}	+	+
10^{-3}	–	+
10^{-4}	–	+

Table 3. Specificity of biotin-labeled PDV cDNA and PPV cDNA capture probes.

Amplified product-source of plant material	Capture probe	Hybrid formation	
		Absorbance at 405 nm	Color development
PDV infected tissue	PDV	2.672	+
PPV infected tissue	PDV	0.021	–
Uninfected tissue	PDV	0.024	–
PDV infected tissue	PPV	0.018	–
PPV infected tissue	PPV	3.536	+
Uninfected tissue	PPV	0.035	–

Absorbance of the above readings were measured at 405 nm. Absorbance for: H_2O = 0.000; PDV in the presence of PPV capture probe = 0.081; PPV in the presence of PDV capture probe = 0.021.

Table 4. Multiplex RT-PCR-ELISA assays for the detection of PDV-B and PPV-D.

PCR annealing temperature (ºC)	PDV capture probe absorbance at 405 nm	PPV capture probe absorbance at 405 nm
60	0.992	3.748
59	0.996	3.545
57	1.141	3.402
55	1.533	3.422
53	1.609	3.646

Simultaneous use of the PDV-specific and PPV-specific capture probes permitted the sensitive detection of both viruses in a multiplex RT-PCR-ELISA assay (Table 4). The annealing temperature of PCR influences the absorbance values of hybrid formation of PDV assay but not of hybrid formation of PPV assay under our experimental conditions.

Nucleotide sequences analyses. Nucleotide sequence analyses of several clones of amplified PDV cDNA and PPV cDNA obtained from nucleic acid extracts of infected leaves from different geographical locations were determined. The sequences obtained were then compared with the coat protein gene sequences available for PDV or with the 3' non-coding region sequences available for PPV. Multiple sequence alignment indicated that the coat protein gene sequences of PDV isolates from Egypt or the U.S. and from peach, plum or cherry share 97% to 98% identity (Fig. 3). Similarly, multiple sequence alignment of the 3' non-coding region of the four strains of PPV showed greater than 90 % identity (not shown) which confirmed previous work (Hadidi and Levy, 1994; Levy and Hadidi, 1994; Nemchinov *et al.*, 1996, 1998), and showed that, indeed, we were working with PPV.

DISCUSSION

Results presented in this report demonstrated the successful use of multiplex RT-PCR-ELISA to directly detect PDV and PPV in nucleic acid extracts from infected tissue and indicated its feasibility as a sensitive rapid laboratory assay for detecting the two viruses. This assay is 10-100 fold more sensitive than detection of PDV and PPV by RT-PCR and analysis of the amplified

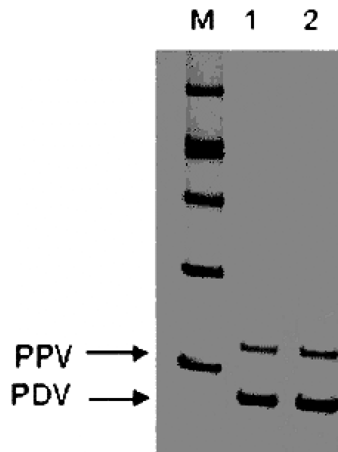

Fig. 2. Gel electrophoretic analysis of multiplex RT-PCR products amplified from Gene-Releaser-treated total RNA mixture from PPV-D-infected and PDV-B-infected leaves (lanes 1 and 2). Molecular DNA marker (M, see Fig. 1), arrows indicate PPVcDNA (220 bp) and PDVcDNA (172 bp) amplified products.

product by gel electrophoresis. About 6 hours are required for positive identification of either PDV or PPV by RT-PCR-ELISA.

Total RNA was extracted from infected or uninfected tissue using commercially available RNA extraction kits, according to the manufacturer's protocol followed by treatment of the extract with GeneReleaser™. Both BIO 101 Fast RNA Green kit and QIAGEN RNeasy kit were efficient in producing viral RNA suitable for RT-PCR and RT-PCR-ELISA assays of PDV and PPV. Thus the commercially available RNA extraction kits may replace the use of organic solvents for RNA extraction. In addition to using the newly harvested tissue, these kits were also used for RNA extraction from frozen and powdered PDV or PPV-infected tissue. These findings make PCR detection studies of PDV or PPV and possibly other fruit tree viruses and viroids easier to conduct.

Primers used for RT-PCR amplification of PDV and PPV have shown previously to be virus specific. (Hadidi *et al.,* 1994; Levy *et al.*, 1994; Parakh *et al.*, 1996). The specificity of the capture probe for each virus in this investigation was established by the lack of detectable hybridization of the capture probe to the amplified cDNA of the other virus and to the amplified products of uninfected tissue. The capture probe hybridized only with its complementary RT-PCR amplified product from virus-infected tissue.

Multiplex RT-PCR-ELISA has been reported for the closely related *Prunus necrotic ringspot virus* and *Apple mosaic virus* (Candresse *et al.*, 1998b). These two viruses show serological cross reactivity (Barbara *et al.*, 1978) and a high level of conservation of the coat protein gene (Candresse *et al.*, 1998b) which suggest that the two pathogens are strains of the same virus. Our investigation is the first to show the successful application of RT-PCR-ELISA for the detection of two unrelated viruses, PDV which belong to the genus *Ilarvirus* and PPV which is a member of the genus *Potyvirus*.

The simultaneous sensitive detection of PDV and PPV (in a single reaction) by multiplex RT-PCR-ELISA should reduce the cost and time required for stone fruit indexing. Besides virus identification, the assay eliminates the use of electrophoresis, thus the use of ethidium bromide or silver nitrate for gel staining is no longer

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PDV,Peach,USA St. ATGGATGCGATGGATAAAATAGTCAGTGGATGACTATATGATCCATCATTTGATTGTGCTTCCACTATGAGTATTCCTAG     80
PDV, Peach, EGYPT -------g-------------------------------------------------a----------------------     80
PDV, Plum, EGYPT  -------g---------n--------------------------g----------------------------------a     80
PDV, Cherry, USA1 -------g------------------------------------g----------------------------------a     80
PDV, Cherry, USA2 -------g---------t-------------------------------------------------------------a     80

PDV,Peach,USA St. GAATATTCGTAGTTGGAAATGCTGCTTTTGCAACAGAATCCACCATTCAGAGTTTGTCACTGAATGTTAAATCCTTTTGG    160
PDV, Peach, EGYPT ----------------------------------g----------------t----------------------------    160
PDV, Plum, EGYPT  ---------------------------------t----------------------------------------------    160
PDV, Cherry, USA1 ------------------------t--------t----------------------------------------------    160
PDV, Cherry, USA2 ---------------------------------t----------------------------------------------    160

PDV,Peach,USA St. TTAACCTGCACTA                                                                      173
PDV, Peach, EGYPT -------------                                                                      173
PDV, Plum, EGYPT  -------------                                                                      173
PDV, Cherry, USA1 -------------                                                                      173
PDV, Cherry, USA2 -------------                                                                      173
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Fig. 3. Multiple alignment of the nucleotide sequence of several clones of amplified PDV cDNA with the corresponding region of published PDV standard sequence (Bachman *et al.,* 1992). Nucleotide sequences of PDV isolates were compared with that of the coat protein gene of PDV RNA 3. The percentage identity of PDV peach or plum isolate from Egypt and cherry isolates from the US was 97%-98% to that of the US PDV peach standard.

needed. In addition, it has advantages of speed, suitability for large number of samples, visual examination and adaptation to automation.

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